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Epac1 regulates TLR4 signaling in the diabetic retinal vasculature

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Abstract

Toll-like receptor 4 (TLR4) polymorphisms occur in diabetic patients. Previous work showed that TLR4 is in the retina of diabetic mice, as well as in retinal endothelial cells (REC) and Müller cells. Since we have shown that exchange protein activated by cAMP 1 (Epac1) can reduce inflammatory mediators, we hypothesized that Epac1 would inhibit TLR4 signaling. We also hypothesized that direct TLR4 inhibition would protect the diabetic retina. Human REC in normal and high glucose were treated with an Epac1 agonist to explore the actions of Epac1 on TLR4 signaling *in vitro*. Subsequently, 2-month diabetic endothelial cell specific knockout mice for Epac1 (Cdh5Cre-Epac1) and Epac1 floxed mice retinas were used for Western blotting for TLR4 signaling pathways. We also used direct inhibition of TLR4 via Tak242 to investigate diabetes-induced changes in retinal permeability and neuronal loss in the mice. The Epac1 agonist reduced TLR4 signaling in REC grown in high glucose. TLR4 levels and both MyD88-dependent and -independent signaling pathways are increased in Cdh5Cre-Epac1 mice compared to Epac1 floxed mice. Tak242 reduced TLR4 signaling in diabetic mice and reduced diabetes-induced increases in permeability and cell loss in the ganglion cell layer in the Epac1 floxed and Cdh5Cre-Epac1 mice. In conclusion, Epac1 reduced TLR4 signaling in the retina and in REC. Direct inhibition of TLR4 was able to protect the retina against diabetes-induced changes in permeability and cell numbers in the ganglion cell layer.

Keywords

Toll-like receptor 4; exchange protein for cAMP 1; retinal endothelial cells; diabetes; signal transduction; inflammation

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1.0 Introduction

It is clear that diabetes induces an inflammatory phenotype in the retina [1–3]. While a number of inflammatory mediators are increased in retinal cells following exposure to high glucose [4, 5], a potential role for the innate immune regulation of these inflammatory factors in diabetic retinopathy is also of interest. Increased levels of TLR2/4 were found in human type 1 diabetic patients with retinopathy compared to patients without retinopathy complications [6]. In addition, numerous studies have shown that TLR4 polymorphisms and haplotypes are involved in diabetic vascular complications and may lead to earlier onset diabetic retinopathy [7, 8]. Studies have extended the human work to the streptozotocin model of type 1 diabetes to demonstrate that diabetes increases TLR4 and downstream signaling pathways, leading to increased C-reactive protein, nuclear factor kappa beta (NFkB) and tumor necrosis factor alpha (TNF*a*) in peritoneal macrophages [9]. The increase in the inflammatory mediators in diabetes was eliminated in global diabetic TLR4 knockout mice [9].

Others demonstrated that high glucose causes increased TLR4 signaling in human retinal endothelial cells (REC) through TLR2/4 [10]. We demonstrated that the β-adrenergic receptor agonist, Compound 49b, inhibited TLR4 signaling in retinal cells [11], which agreed with work on β-adrenergic effects on TLR4 in other tissues. Downstream of β-adrenergic receptors are protein kinase A (PKA) and exchange protein for cAMP 1 (Epac1).

Epac1 is ubiquitously expressed [12], with both Epac1 and Epac2 localized in the eye [13]. Epac1 was reported to be involved in endothelial cell permeability in other cell types [14]. We have previously reported that Epac1 is more involved in retinal responses when compare to Epac 2 [11]. Therefore, we investigated whether Epac1 could reduce inflammatory mediators in the retinal vasculature [11], as well as protect against diabetes-induced damage [15]. We also showed that Epac1 endothelial cell specific knockout mice have reduced TLR4 levels in whole retinal lysates [16].

In the present study, we hypothesized that Epac1 would block TLR4 signaling in retinal endothelial cells and in diabetic retinal lysates. We also hypothesized that direct inhibition of TLR4 would protect against diabetes-induced increases in permeability and loss of cell number in the retinal ganglion cell layer (RGC). We used endothelial cell specific knockout (KO) mice for Epac1, as well as human REC in culture to investigate this hypothesis.

Experimental Procedures

Epac1 floxed and cdh5 Cre mice.

All animal procedures meet the Association for Research in Vision and Ophthalmology requirements and were approved by the Institutional Animal Care and Use Committee of Wayne State University (Protocol 18-03-0575) and conform to NIH guidelines. Epac1 floxed mice (B6;129S2-Rapgef3$^{tm1Geno/J}$ mice) and B6 FVB-Tg (cdh5-cre)7Mlia/J Cre mice were purchased from Jackson Laboratories and bred to generate conditional knockout mice in which Epac1 is eliminated in vascular endothelial cells. Both male and female Epac1 floxed and cdh5Cre-Epac1 mice were used for these experiments at 2 months of age [11, 17]. Mice

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were on a standard light/dark cycle and allowed food *ad libitium*. All mice were randomly assigned into an experimental group for control, diabetes or Tak242 treatment.

Diabetes was induced by 60 mg/kg intraperitoneal injections of streptozotocin dissolved in citrate buffer for up to five consecutive days in Epac1 floxed and Cdh5Cre-Epac1 mice at the same time each day in the vivarium. Glucose measurements were done each week, with glucose levels >250 mg/dL accepted as diabetic. Mice were not fasted before glucose measurements, and all measurements were taken on ~5 μL blood samples measured by a handheld measurement device and taken in the vivarium. Table 1 provides body weights and glucose measurements from all mice.

Some diabetic Epac1 floxed and Cdh5Cre-Epac1 mice were treated with Tak242, a TLR4 inhibitor. Tak242 (3mg/kg/day, Tocris Bioscience, UK) was dissolved in the drinking water for 2 months. Water consumption was measured each day to insure the mice consumed the Tak242. After 2 months of treatment, mice were euthanized for measurement of changes in permeability, retinal thickness, and protein levels. Euthanasia on all mice was performed using CO2 followed by cervical dislocation.

Retinal Endothelial Cell Culture.

Primary human retinal endothelial cells (REC) were purchased from Cell Systems Corporation (CSC, Kirkland, Washington) and grown in Normal Glucose Cell Systems medium supplemented with microvascular growth factors (MVGS), 10μg/mL gentamycin, and 0.25μg/mL amphotericin B (Invitrogen, Carlsbad, CA). Once cells reached confluence, some dishes were moved to Cell Systems High Glucose Medium (25 mM glucose). All cells were cultured on attachment factor coated dishes. Only cells up to passage 6 were used. Cells were quiesced by incubating in high or normal glucose medium without MVGS for 24 h prior to experimental use. Some cells in both normal and high glucose were treated with an Epac1 agonist (8-CPT-2'-O-Me-cAMP, 10μM, 24 hours [18].

Measurements of permeability.—Analyses of vascular leakage were done on all mice at 2 months of diabetes and Tak242 treatment. Mice were transfused with 200ul Evans blue (0.5% in saline, Sigma Aldrich) via the tail vein. Forty-five minutes after infusion, mice were euthanized, retinas were removed, placed into 100ul formamide, and incubated for 48hours at 55°C. Tubes were then centrifuged and transferred to a 96 well plate. The absorbance of the retina was measured at 610 [19]. For these studies, experiments were initiated with 10 mice in each group and retinas pooled into 1 tube, and each group had an N=5–7 for analyses.

Neuronal measurements.

For these studies, we used 4 mice in the Epac1 floxed and CreLox alone or with STZ. Six mice per group were used for mice receiving Tak242. At 2 months of diabetes or diabetes +Tak242 treatment, a subset of each group of mice was sacrificed for measurements of neuronal thickness, as we have previously published [20]. Ten micrometer sections were taken from regions throughout the retina and every 10^{th} section was analyzed for measurement. Analyses of retinal thickness and cell numbers in the RGC were assessed

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from the same regions in each retina, as we have done in the past [21, 22]. Analyses were not done in a blinded fashion.

Western blotting.

Whole retinal lysates or cell culture lysates were collected into lysis buffer containing protease and phosphatase inhibitors. For mouse samples, 2 retinas from 1 mouse were pooled together from 5 mice in each group. Equal amounts of protein were separated using pre-cast tris-glycine gels (Invitrogen, Carlsbad, CA), and blotted onto nitrocellulose membranes. After blocking in TBST (10mM Tris-HCl buffer, pH 8.0, 150 mM NaCl, 0.1% Tween 20) and 5% (w/v) BSA, membranes were treated with a Epac1, TLR4, MyD88, IL-1β, TNFα, IRAK, TRAF6, IRF3, phosphorylated and total NFkB antibodies (Abcam, Cambridge, MA), and beta actin (Santa Cruz Biotechnology, Santa Cruz, CA) primary antibodies followed by incubation with secondary antibodies labeled with horseradish peroxidase. Antigen-antibody complexes were visualized using a chemilluminescence reagent kit (Thermo Scientific, Pittsburgh, PA) and data was acquired with an Azure C500 machine (Azure Biosystems, Dublin, CA). Western blot band densities were measured using Image Studio Lite software.

Statistics.

Data are presented as mean ±SD. We used 1-way ANOVA for statistical analyses with Tukey's post hoc tests. $P<0.05$ was accepted as significant. Data was analyzed using Prism 7.0 (GraphPad software). The protein data show representative blots of key proteins cropped from whole blots. For the Western blot data, 2 retina from the same mouse were pooled into 1 tubes and 5 mice/group were analyzed for data. Retina were pooled for the Evan's Blue permeability analyses.

Results

Epac1 reduces TLR4 signaling in retinal endothelial cells grown in high glucose.

We initially wanted to investigate Epac1 actions on TLR4-MyD88-dependent and -independent signaling in human REC in culture. REC were grown in normal glucose or high glucose and treated with an Epac agonist. We performed Western blotting for proteins downstream of TLR4 in the MyD88-dependent and -independent pathways. Figure 1F confirms that the Epac1 agonist worked to increase Epac1 levels, serving as a control [15]. Panels 1B-E demonstrate that REC grown in high glucose have increased MyD88-dependent and -independent signaling, which is eliminated in cells treated with the Epac1 agonist, suggesting that Epac1 is upstream of TLR4-dependent signaling.

Tak242 reduced TLR4 levels in the diabetic retina.

We expanded cell culture data to explore diabetes in Epac1 floxed and Cdh5Cre-Epac1 mice. Figure 2A shows that loss of Epac1 in the endothelial cells reduces Epac1 levels in whole retinal lysates. Diabetes significantly reduced Epac1 levels. We also treated the diabetic Epac1 floxed and Cdh5Cre-Epac1 mice with the TLR4 inhibitor, Tak242. Figure 2B demonstrates that oral delivery of Tak242 was effective in reducing TLR4 levels in the

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diabetic retina of both Epac1 floxed and Cdh5Cre- Epac1 mice. Diabetes increased TLR4 levels in both Epac1 floxed and Cdh5-Epac1 mice.

Loss of Epac1 increased both MyD88-dependent and -independent signaling in the diabetic retina, while TLR4 inhibition reduced both signaling pathways.

We previously reported that Compound 49b, a novel β-adrenergic receptor agonist, reduced TLR4 levels in retinal endothelial cells (REC), Müller cells, and whole retinal lysates [10]. We wanted to expand those findings to determine if Epac1, which lies downstream of β-adrenergic receptors, could reduce TLR4 signaling pathways, both MyD88-dependent and MyD88-independent. Figure 3 shows that loss of Epac1 in endothelial cells significantly increased levels of the TLR4 signaling pathway members studied (MyD88, IRAK1, TRAF6, TRAM1, IRF3), suggesting that Epac1 regulates TLR4 signaling. Additionally, we used Tak242 to directly block TLR4 signaling in diabetic Epac1 floxed and Cdh5Cre- Epac1 mice. In both sets of diabetic mice, Tak242 was able to inhibit diabetes-induced increases in both MyD88-dependent and -independent signaling, irrespective of the presence of Epac1 (Figure 3).

TLR4 inhibition reduces diabetes-induced increases in inflammatory mediators.

Loss of Epac1 in endothelial cells from the mice increased NFkB phosphorylation (A), TNFα (B), and IL-1β (C) levels in whole retinal lysates (Figure 4). Diabetes increased these inflammatory mediators, which were significantly reduced by the TLR4 antagonist. Taken with Figure 3, Epac1 regulated TLR4 signaling, and direct inhibition of TLR4 was also effective at reducing diabetes-induced protein changes.

TLR4 inhibition reduced diabetes-induced increases in retinal permeability.

Diabetes was induced in the Epac1 floxed and Cdh5Cre-Epac1 mice and led to a significant increase in permeability (Figure 5). Tak242 in the drinking water was able to significantly reduce the diabetes-induced increase in retinal permeability. These data agree well with our previously findings using the ischemia/reperfusion model of retinal stressors [23].

Inhibition of TLR4 reduces the diabetes-induced loss of cell numbers in the ganglion cell layer, but does not improve retinal thickness.

The most commonly reported neuronal changes reported in the diabetic retina at 2 months are loss of cell numbers in the retinal ganglion cell layer (RGC) and loss of retinal thickness. We have previously reported no differences in these measurements in non-diabetic Epac1 floxed vs. Cdh5Cre- Epac1 mice [15]. Diabetes reduced these measurements in both groups of mice (Figure 6). Inhibition of TLR4 by Tak242 rescued the loss of cell number in the RGC, but did not improve retinal thickness in the diabetic animals (Figure 6).

Discussion.

In the past decade, the role of innate immunity in diabetic retinopathy has become of interest [3, 6]. Literature suggested that polymorphisms of TLR4 were observed in patients with proliferative diabetic retinopathy [7]. Additionally, work in TLR4 knockout mice showed protection against diabetic with significantly reduced inflammatory markers [9]. We have

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previously reported that Compound 49b, a novel β-adrenergic receptor agonist, significantly reduced TLR4 levels in diabetic mice, REC and Müller cells [10]. In addition to β-adrenergic signaling, studies have shown that other G-protein signaling pathways may play a role in diabetic retinopathy [24, 25]. We also showed that Epac1 reduced inflammatory mediators in retinal endothelial cells [11], suggesting that it is downstream signaling of β-adrenergic receptors and may mediate the anti-inflammatory response in endothelial cells. Based on our previous work, these studies sought to explore whether Epac1 could work upstream of TLR4 to inhibit MyD88-dependent and -independent signaling in the mouse vasculature, as well as determine if direct inhibition of TLR4 could reduce diabetes-induced increases in permeability and neuronal loss.

Our data showing that Epac1 lowered TLR4 levels and signaling pathways agrees with literature using CD-1 mice treated with Paeoniflorin, a monoterpene glycoside, which demonstrated that treatment significantly reduced high glucose-mediated TLR4/NFkB signaling in microglia [26]. Other work in streptozotocin-treated rats showed increased TLR4/MyD88 signaling [27], which we observed in the Epac1 Cdh5Cre-Epac1 mice [16]. We focused these studies on Epac1 instead of Epac2, as we have previously reported that Epac1 drives much of the high glucose response on retinal endothelial cells [11]. In addition to Epac1, other factors could be involved, such as human antigen R (HuR), vascular endothelial cell growth factor (VEGF) and PKCbeta [28, 29].

However, we have chosen to focus on Epac1 based upon our previous work showing that Epac1 regulated TLR4 [16]. Epac1 regulation of TLR4 signaling may be cell-type specific, suggesting that increased understanding of Epac1 actions in the retina is key to allowing for novel drug development for diabetic retinopathy. Additionally, Cdh5 Cre can also target leukocytes, known to be involved in diabetic damage. We cannot rule out that some of the responses noted are from leukocyte actions in the retina; however, our cell culture data suggests that the retinal vasculature is definitely involved. The role of endothelium vs. leukocyte in TLR4 regulation will be studied in the future. We expected to find a difference in neuronal and permeability changes in the diabetic cdh5-Cre Epac1 mice vs. Epac1 floxed mice. While we did find Epac1 regulation of TLR4 signaling proteins, we did not find protection against diabetes-induced changes in the Epac1 floxed mice. This suggests it likely requires more than changes in the Epac1 levels in retinal endothelial cells to offer retinal protection against diabetes. This will be explored further in the future. While we find a protective effect of Epac1 on TLR4 signaling, Epac1 has been associated with some angiogenic potential in other models [30, 31]. Thus, we will continue these studies to evaluate the role of Epac1 in angiogenic properties in retinal endothelial cells.

In the present work, we demonstrate that Tak242, a TLR4 inhibitor, was able to reduce diabetes- induced increases in permeability, which follows what has been reported in other models [27]. Tak242 was administered in the drinking water, suggesting that treatment may be applicable to development as an oral therapy. We also found that oral Tak242 reduced diabetes-induced of cells in the retinal ganglion cell (RGC) layer, but not retinal thickness. Our findings on protection of retinal ganglion cells agrees with studies done in the optic nerve crush model [32]. We did not see changes in whole retinal thickness after Tak242, which follows other studies in global TLR4 KO mice showing limited structural differences

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in photoreceptor layers [33]. Others have also reported that pharmacological inhibition of TLR4 did not alter overall thickness of the retina [34]. These findings from the literature agree with the data in the current study. These changes in RGC could also be attributed to changes in trophic factors [24], which can be studied in future work. Future studies will also continue the studies with Tak242 to investigate whether direct TLR4 inhibition can reduce vascular changes, and structure/functional differences. At this point, it was cost-prohibitive and difficult to obtain suitable concentrations of Tak242 for oral delivery for longer studies.

Conclusions.

There are 2 main conclusions from this study. First, loss of Epac1 in endothelial cells increased TLR4 signaling (both MyD88-dependent and -independent) in non-diabetic whole retina lysates. Secondly, direct inhibition of TLR4 was able to prevent short-term diabetes-induced changes in retinal permeability and cell loss in the ganglion cell layer, as Tak242 reduced retinal leakage and prevented the loss cells observed in diabetes at 2 months. Retinal thickness was not altered by pharmacological oral inhibition of TLR4. Taken together, development of Epac1 agonist may be used to block TLR4 actions in the retina. Additionally, TLR4 antagonists could offer new directions for therapy development.

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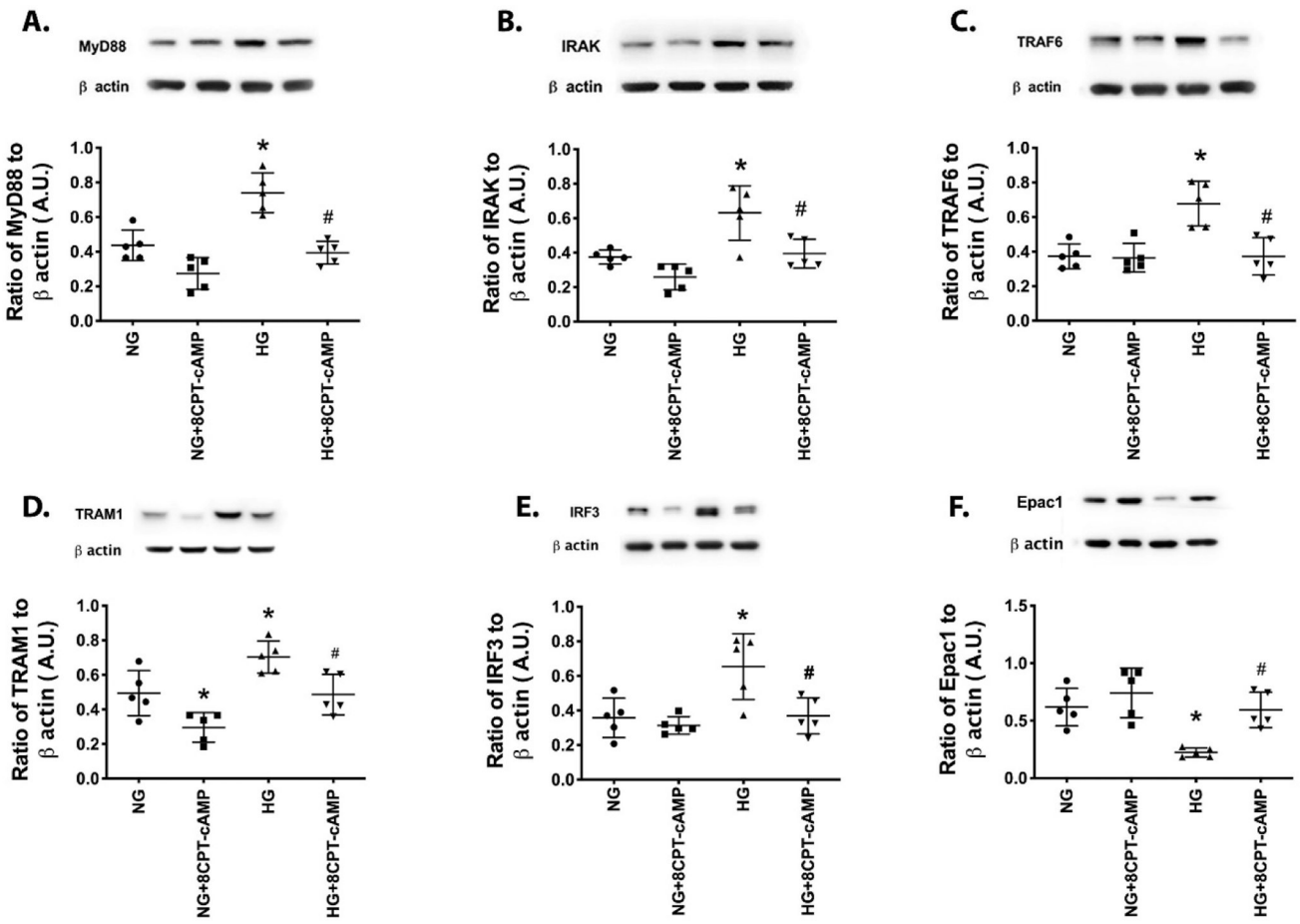

Figure 1.

Epac1 agonist regulates MyD88 signaling in human retinal endothelial cells. Retinal endothelial cells were grown in normal glucose (NG) or high glucose (HG) and treated with an Epac1 agonist. Western blotting was done for MyD88 (A), IRAK1 (B), TRAF6 (C), TRIM1 (D), IRF3 (E), and Epac1 (F). Representative blots are shown. *P<0.05 vs. NG, #P<0.05 vs. HG. N=5 for all groups. Data are mean±SD.

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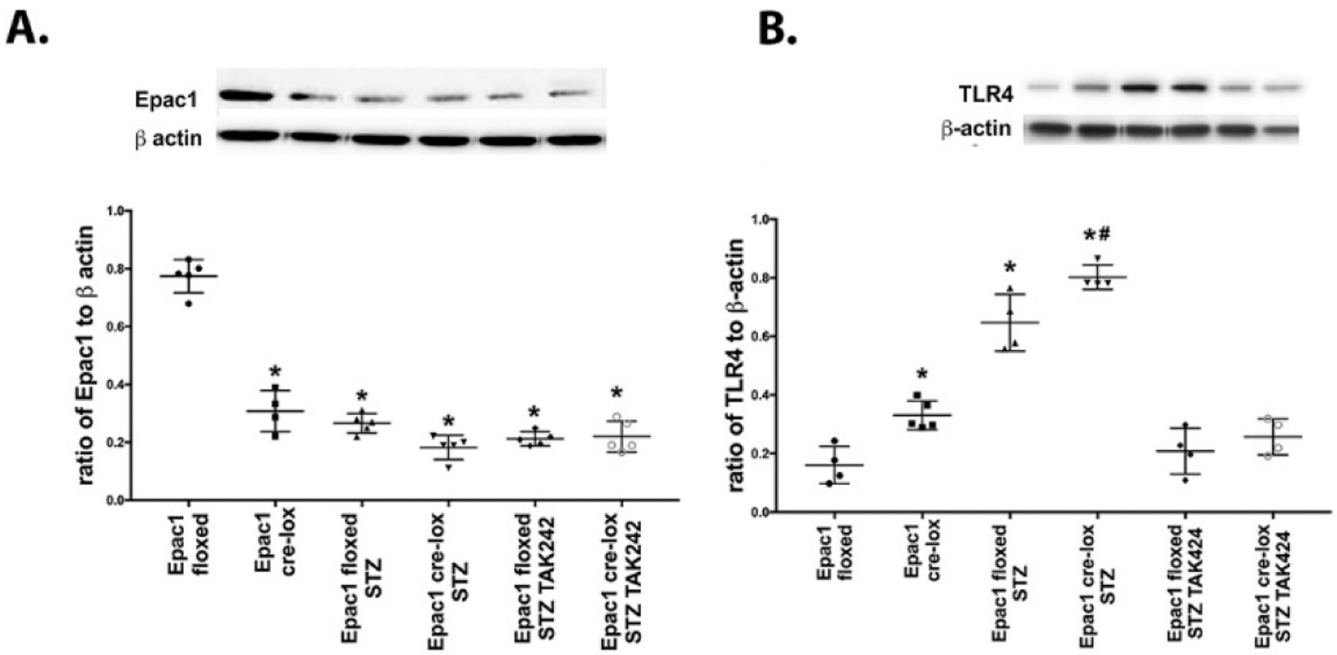

Figure 2.

Tak242 reduced TLR4 levels in whole retinal lysates. Some Epac1 floxed and Cdh5Cre-Epac1 mice were made diabetic and others were made diabetic and given Tak242 in the drinking water. Panel A shows Epac1 levels to show successful elimination of Epac1 in the mice, while Panel B is TLR4 levels to show that Tak242 was effective. *P<0.05 vs. Epac1 floxed. #P<0.05 vs. Epac1 floxed+STZ. N=5 for all groups except Epac Crelox where N=4. Data are mean±SD.

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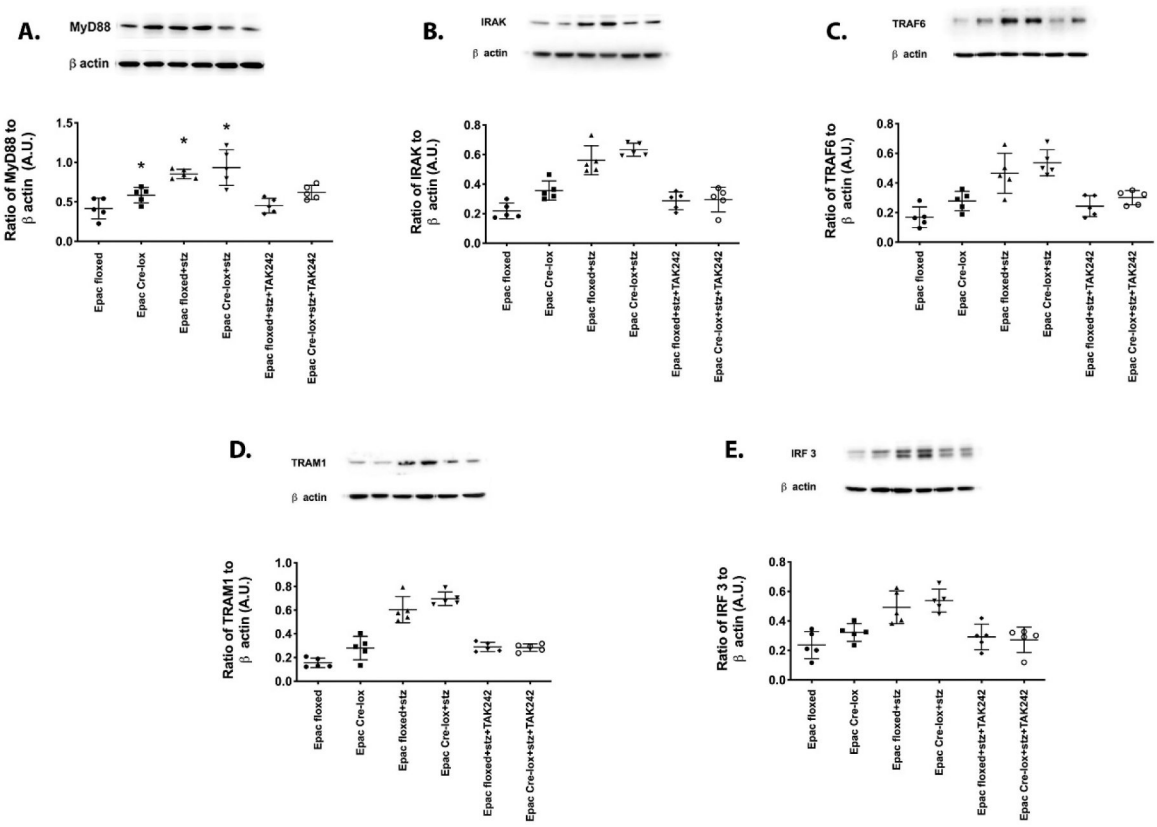

Figure 3.

Loss of Epac1 increased both MyD88-dependent and -independent signaling. Some Epac1 floxed and Cdh5Cre-Epac1 mice were made diabetic and others were made diabetic and given Tak242 in the drinking water. Data are from mice at 2 months of diabetes and Tak242 treatment. Panel A is MyD88, Panel B is IRAK1, and Panel C is TRAF6 of the MyD88-dependent pathway. Panel D is TRAM1 and Panel E is IRF3 of the MyD88-independent pathway. *P<0.05 vs Epac floxed compared to all, # P<0.05 of Epac floxed +STZ compared to Epac floxed+stz+tak242, and $ P<0.05 for Cdh5Cre- Epac1 +stz compared to Cdh5Cre-Epac+stz+tak242. N=5 for all groups. Data are mean±SD.

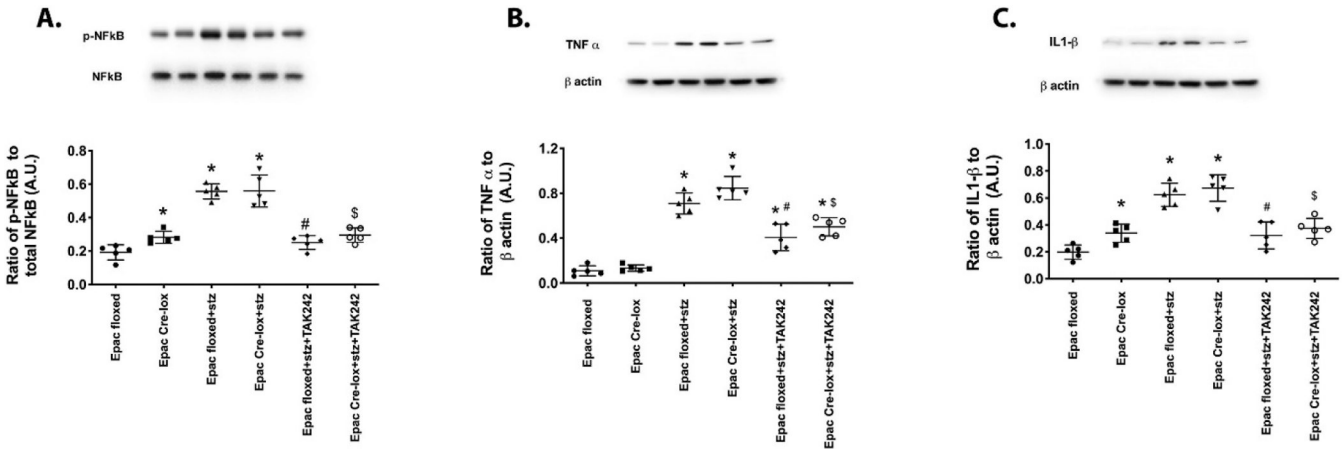

Figure 4.

Loss of Epac1 increased inflammatory mediators, while Tak242 blocked diabetes-induced increases in inflammatory mediators. Some Epac1 floxed and Cdh5Cre-Epac1 mice were made diabetic and others were made diabetic and given Tak242 in the drinking water. Data are from mice at 2 months of diabetes and Tak242 treatment. Panel A is phosphorylated NFkB to total NFkB, Panel B is TNF*a*, and Panel C is IL-1*b*. *P<0.05 vs Epac floxed compared to all, # P<0.05 of Epac floxed +STZ compared to Epac floxed+stz+tak242, and $ P<0.05 for Cdh5Cre-Epac1 +stz compared to Cdh5Cre-Epac +stz+tak242. N=5 for all groups. Data are mean±SD.

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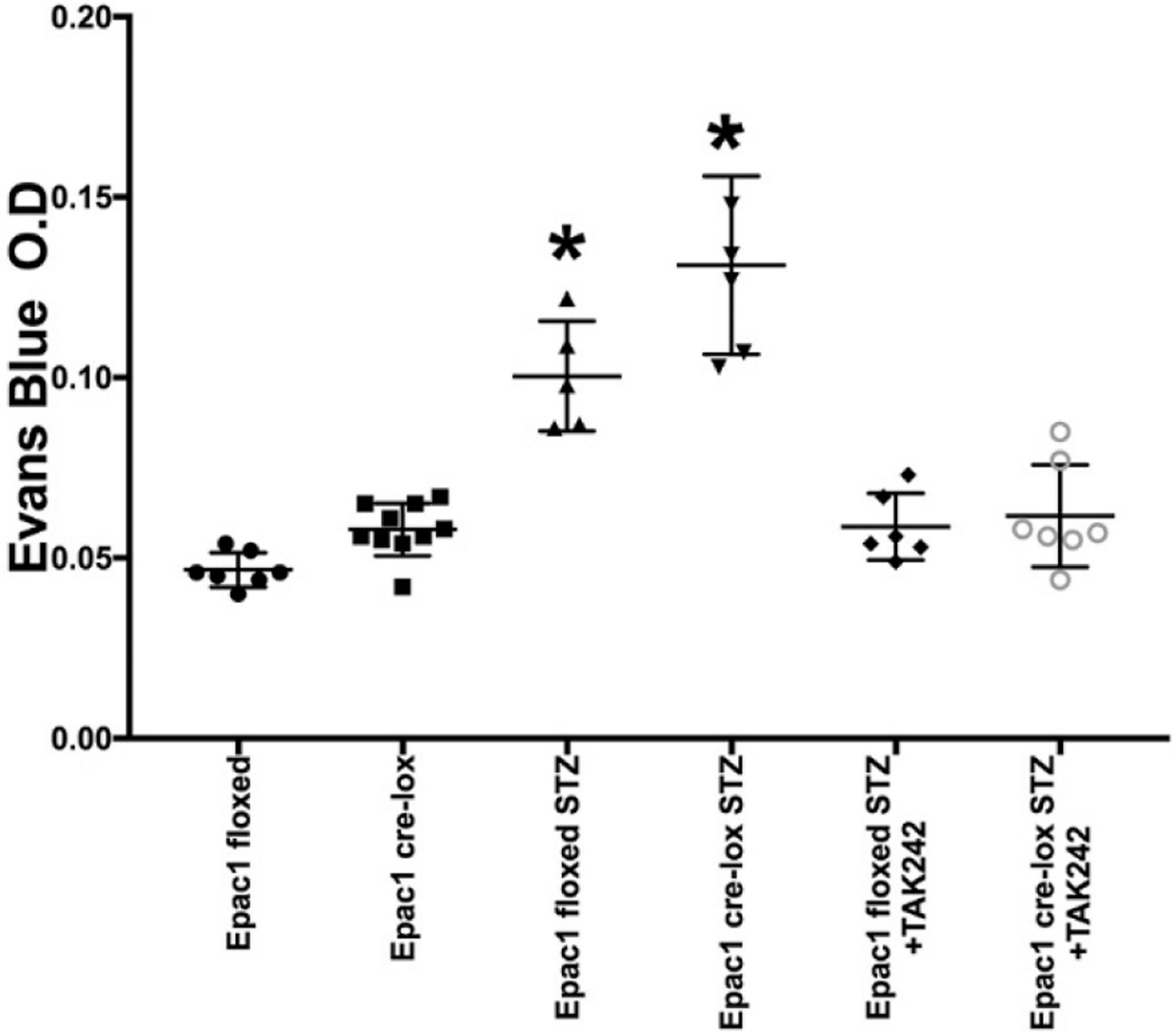

Figure 5.

TLR4 antagonist reduced permeability. Some Epac1 floxed and Cdh5Cre-Epac1 mice were made diabetic and others were made diabetic and given Tak242 in the drinking water. *P<0.05 vs. non-diabetic, #P<0.05 vs. diabetic Epac1 floxed, $P<0.05 vs. diabetic Cdh5Cre-Epac1 mice. N=8 for Epac and EpacCrelox, N=5 for Epac+STZ and Epac Crelox+STZ, N=6 for Epac floxed+STZ+Tak242, and N=7 for Epac CreLox+STZ+Tak242. Data are mean ±SD.

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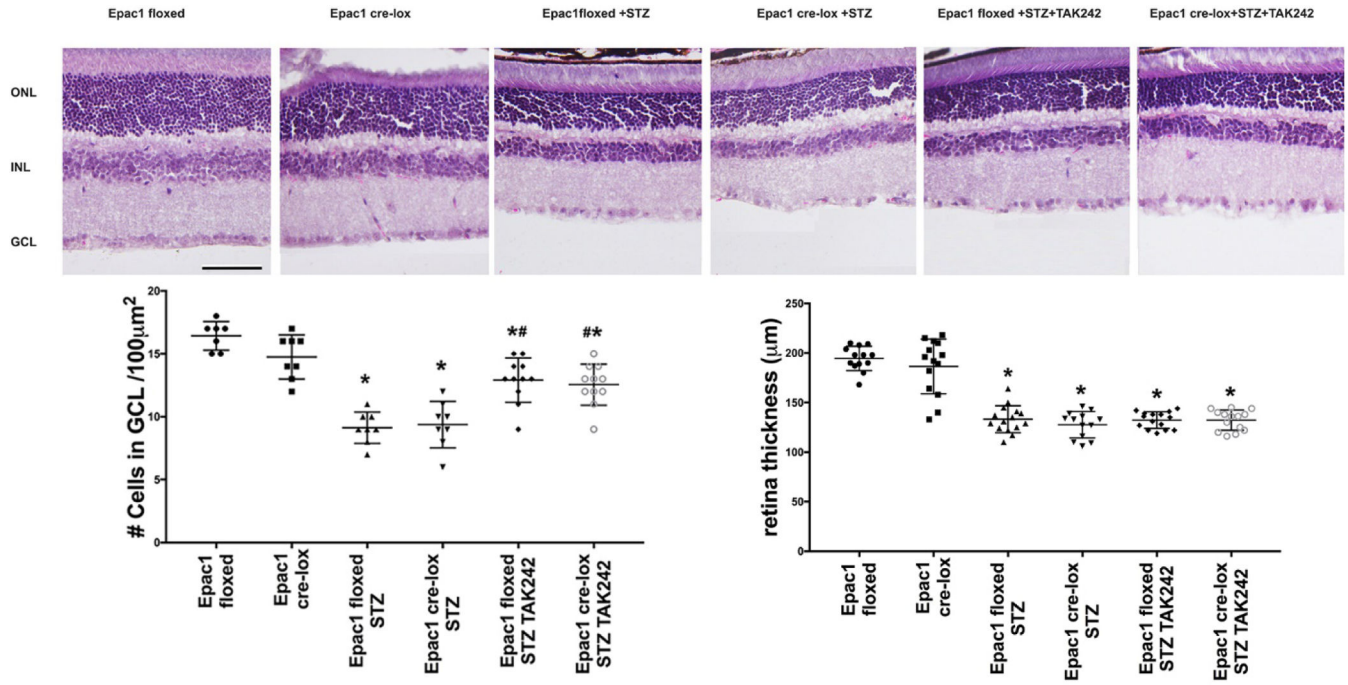

Figure 6.

TLR4 inhibition reduced diabetes-induced loss of cells in the retinal ganglion cell layer. Some Epac1 floxed and Cdh5Cre-Epac1 (Epac CreLox) mice were made diabetic and others were made diabetic and given Tak242 in the drinking water. Top panels are images of the retinal layers. The left bar graph is number of cells in the ganglion cell layers, and the right graph is retinal thickness. *P<0.05 vs. non-diabetic, , #P<0.05 vs. diabetic Epac1 floxed or $P<0.05 vs. diabetic Cdh5Cre-Epac1 (Epac CreLox) mice. N=7 for Epac floxed, Epac1 CreLox, and both groups+STZ and N=10 for both groups +STZ+Tak242 for RGC measurement. For retinal thickness, N=13 measurements for Epac floxed, Epac1 CreLox, and both groups+STZ, and N= 16 for both groups +STZ+Tak242 for RGC measurement. Data are mean ± SD. Scale bar is 50um.

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Table 1.

Data are mean±SD.

	Epac1 floxed						Cdh5Cre-Epac1					
	Epac1 floxed		Epac1 floxed STZ		Epac1 floxed STZ TAK242		Epac1 cre-lox		Epac1 cre-lox STZ		Epac1 cre-lox STZ TAK242	
	BW (g)	BG (mg / dL)	BW (g)	BG (mg / dL)	BW (g)	BG (mg / dL)	BW (g)	BG (mg / dL)	BW (g)	BG (mg / dL)	BW (g)	BG (mg / dL)
Before STZ	26.5±1.7	112±10	27.6±5.2	109±11	26.6±2.1	105±2.2	24±1.5	121±9	27.5±1.2	118±12	26.3±1.7	113±9
2mon after STZ	30±2.5	121±13	24.4±3.7*	411±66#	23.8±1.9*	429±6.8#	29±2.9	128±10	25.5±2.4*	383±84#	23.3±1.9*	417±59#

*p<0.05 vs non-diabetic for body weight (BW),

#p<0.05 Vs. non- diabetic for blood glucose (BG).